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Focused ion beam milling scanning electron microscopy (FIB-SEM)

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An abbreviated version of this protocol was published in Science in Dec 2022

Endosomal lipid signaling reshapes the endoplasmic reticulum to control mitochondrial function

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Detailed protocol

Dear Oleg,
Thank you for your interest and I am sorry for a little delay.
As requested, I describe alignment and segmentation workflow:

Alignment:

1. In order to ease alignment we have used running fiducials. Before autoslice and view workflow, we have deposited thin protective layer with carbon onto our cells (300nm). On the top of that, we have deposited fine lines of platinum x=400nm, y=300nm, ionbeam deposition at 40pA and 30kV. On the top of lines (3 to 6 lines) we have deposited the rest of protective carbon layer, 800nm at least. Thus on every cross section during slice and view cycle, there will be seen high contrast little platinum squares in the protective layer.
2. Resulting tif.images were fused into a stack and saved as nrrd file format (using Image J). This allows easier working with stacks above 4GB.
3. Stack was loaded into Microscopy image browser. Great tool with great youtube video tutorials produced by its developer Ilya Belevich.
4. Running fiducial can be selected by magic wand tool (check up 3D box for automatic selection in all serial sections). Go through the stack and check if running fiducial (platinum line cross section in the protective layer) is selected in all the images. Repeat for all running fiducials
5. Open alignment tool window from the dataset menu, check drift correction algorithm, option for “selection”, press continue till aligned.
6. Correct voxel size parameters according to your acquisition settings.
7. Bin the voxel size till isotropic size (10 nm in our case, however it is optional).

Deep Learning segmentation for mitochondria:

1. Chop the volume into small pieces in order to generate small volume/volumes for manual segmentation in order to create ground truth. (Chopping tool in MIB).
2. Take one such mini volumes and segment all mitochondria using brush tool, when possible use interpolate bouton between 5 to 10 sections in order to speed up painting everything. It took around 2 days for me, but smaller volume would have also worked.
3. Save model of manually segmented mitochondria as default matlab file.
4. Watch https://www.youtube.com/watch?v=U5nhbRODvqU
5. Create folder Images and put raw volume into it. Create folder Labels and put matlab file of segmented mitochondria into it.
6. Run deep learning work flow for 3D Unet in MIB. Standard parameters worked for me.
7. Deep learning took 4,5 hours at an average workstation with NVIDIA GPU. Can be run over night.
8. Save neuronal network file.
9. In deep learning workflow of MIB, use generated network file to predict (autosegment) mitochondria in FIBSEM volumes of interest.
10. Using object picker, check if each mitochondria is adequately segmented and is not falsely connected to neighboring mitochondria. Delete false connections if present, delete other structures that have been falsely assigned (for example some lipid droplets). Such model refinement of cellular volume or roughly 1000 cubic micrometers with 60-300 mitochondria has taken 1 working day, that is incomparably little compared to segmenting all mitochondria by hand.
11. Resulting model can be saved as series of TIFF files and opened in any 3D software, such as Imaris, DragonFly, Amira, 3D Slicer etc. We have used Imaris for visualization.

Segmenting ER:

1. Segmented mitochondrial were converted into “mask” in MIB. Mask was inverted to limit father actions to everything but mitochondria. Dilate/Erode could be used in order better shape mitochondria exclusion area and limit overflow between ER and mitochondria outer membrane in resulting model.
2. Global threshoulding tools in MIB were applied (entropy) in order to select higher contrasted objects. Since Mitochondria were excluded, only ER membranes, ribosomes, lipid droplets, Lysosomes, Golgi, some cytoskeletal elements were selected by the global threshoulding. Add it to another material.
3. Lipid droplets, lysosomes, Golgi area could have been easily deleted from material by fast manual painting with a brush tool with heavy use of interpolation tool.
4. Run object statistics. Delete all the small objects like ribosomes, cytoskeletal elements, small vesicles.
5. One ends up with a single big object, that is ER.
6. Export model as series of TIFF files and visualize in 3D software of your choice.

In the end the best guidance are the original manuals of Ilya Belevich, http://mib.helsinki.fi/documentation.html
Great job from him for such a wonderful open source tool.
But please feel free to ask me for further details if needed.
Sincerely, Dmytro Puchkov.

How to cite:(Readers should cite both the Bio-protocol preprint and the original research article where this protocol was used)

1. Jang, W. and Haucke, V. (2023). Focused ion beam milling scanning electron microscopy (FIB-SEM). Bio-protocol Preprint. bio-protocol.org/prep2204.
2. Jang, W., Puchkov, D., Samsó, P., Liang, Y., Nadler-Holly, M., Sigrist, S. J., Kintscher, U., Liu, F., Mamchaoui, K., Mouly, V. and Haucke, V. (2022). Endosomal lipid signaling reshapes the endoplasmic reticulum to control mitochondrial function. Science 378(6625). DOI: 10.1126/science.abq5209